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Side-chain and backbone amide bond requirements for glycopeptide stimulation of T-cells obtained in a mouse model for rheumatoid arthritis

Lotta Holm,[a] Robert Bockermann,[b] Erik Wellner,[a] Johan Bäcklund,[b] Rikard Holmdahl[b] and Jan Kihlberg[a,c,*]

[a]*Organic Chemistry, Department of Chemistry, Umeå University, SE-901 87 Umeå, Sweden*
[b]*Section of Medical Inflammation Research, Lund University, Sölvegatan 19, I11 BMC, SE-221 84 Lund, Sweden*
[c]*AstraZeneca R&D Mölndal, SE-431 83 Mölndal, Sweden*

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Abstract—Collagen induced arthritis (CIA) is the most studied animal model for rheumatoid arthritis and is associated with the MHC class II molecule A^q. T-cell recognition of a peptide from type II collagen, CII256–270, bound to A^q is a requirement for development of CIA. Lysine 264 is the major T-cell recognition site of CII256–270 and CIA is in particular associated with recognition of lysine 264 after posttranslational hydroxylation and subsequent attachment of a β-D-galactopyranosyl moiety. In this paper we have studied the structural requirements of collagenous glycopeptides required for T-cell stimulation, as an extension of earlier studies of the recognition of the galactose moiety. Synthesis and evaluation of alanine substituted glycopeptides revealed that there are T-cells that only recognise the galactosylated hydroxylysine 264, and no other amino acid side chains in the peptide. Other T-cells also require glutamic acid 266 as a T-cell contact point. Introduction of a methylene ether isostere instead of the amide bond between residues 260 and 261 allowed weaker recognition by some, but not all, of the T-cells. Altogether, these results allowed us to propose a model for glycopeptide recognition by the T-cells, where recognition from one or the other side of the galactose moiety could explain the different binding patterns of the T-cells.

1. Introduction

Rheumatoid arthritis (RA) is an autoimmune inflammatory disease affecting approximately 0.5–1% of the population of the industrialised world. Autoimmune diseases develop when the immune system fails to distinguish between self and non-self and in RA this appears as a tissue-specific attack on synovial joints. The symptoms include swelling, stiffness and pain in the joints and subsequent erosion of the underlying bone.[1] The cause of RA is unknown and although there are several symptomatic treatments, there is no cure. In severely affected RA patients both auto-reactive T-cells[2–4] and antibodies[5–7] directed against type II collagen (CII) have been found. CII is a large triple helical protein that is the most abundant protein in cartilage. Individuals that are carriers of the class II major histocompatibility complex (MHC) molecules DR4 and DR1, sharing a common peptide binding pocket, are at larger risk of developing arthritis.[8–10]

Collagen induced arthritis (CIA) is the most common animal model for RA. Following immunization of mice[11] or rats[12] with heterologous or autologous type II collagen the animals develop CIA with symptoms similar to RA. In mice the susceptibility of the disease is associated with the mouse MHC class II molecule A^q.[13,14] In contrast the A^p molecule which only differs in four amino acids as compared to A^q does not confer susceptibility to CIA.[15,16] After immunization the collagen is degraded into peptides by antigen presenting cells (APC). Some of these peptides are then bound to A^q MHC class II molecules and presented to MHC class II restricted αβ T-cells, an event that may activate the T-cell to initiate a response towards the antigen and subsequently to the development of arthritis. A peptide

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* Corresponding author. Tel.: +46 90 7866890; fax: +46 90 138885; e-mail: jan.kihlberg@chem.umu.se

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from the CII molecule, consisting of residues 256–270, is responsible for the T-cell activation in mice expressing A^q molecules.[17] Further studies revealed the minimal epitope, capable of binding to A^q as well as stimulating T-cells, to consist only of amino acids 260–267 (Fig. 1).[18] Within the minimal epitope, the side chains of isoleucine at position 260 and phenylalanine at position 263 have been found to be important P1 and P4 anchor positions for binding to the A^q molecule.[19,20] The only position that differs between mice and other species (rat, bovine, chicken and human) within the immunodominant epitope is position 266, where the glutamic acid replaces the aspartic acid found in the mouse. The mouse peptide (Asp266) has a lower binding affinity to A^q and is less antigenic, possibly because of the lower affinity.[17]

Many of the lysine residues in CII are variably post-translationally hydroxylated and further glycosylated with a β-D-galactopyranosyl- or an α-D-glucopyranosyl-(1 → 2)-β-D-galactopyranosyl unit.[21] This is the case for lysine 264, situated in the centre of the immunodominant peptide. In fact most CII-reactive T-cell hybridomas obtained from mice developing CIA (20 out of a panel of 29 hybridomas) specifically recognise the β-D-galactopyranosyl unit at this position.[22–24] For T-cell recognition of the naked peptide, with a non-modified lysine 264, several of the amino acid side chains, for example, positions 261, 262, 264, 266 and 267, show varying importance as interaction sites for different T-cell clones with lysine 264 and glutamic acid 266 playing the essential roles.[19,25,22] The carbohydrate dependent hybridomas have been found to require specific hydroxyl groups of the β-D-galactopyranosyl unit in order to trigger a response, and could be subdivided into four

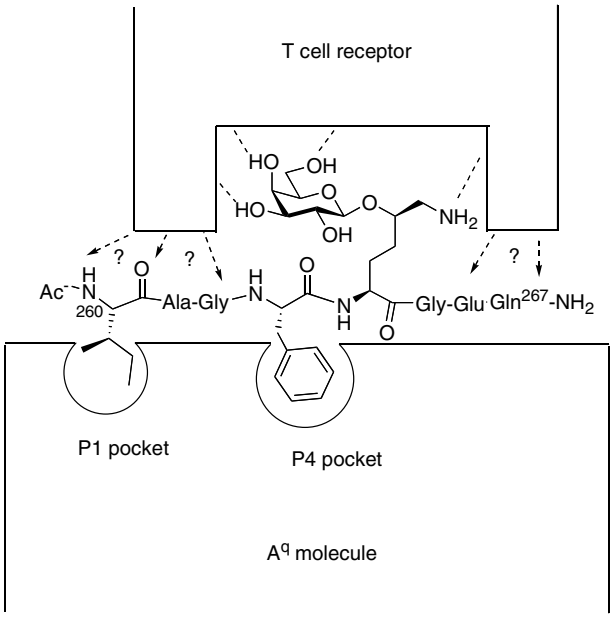


Figure 1. The minimal collagen glycopeptide CII260–267 is anchored by residues isoleucine 260 and phenylalanine 263 to the MHC class II molecule A^q. Most T-cells recognise the galactosylated hydroxylysine 264 at the centre of the epitope. In this paper the requirements of the peptide for eliciting a response from T-cell hybridomas are investigated.

different groups depending on this fine specificity.[26,27] Interestingly, vaccination of mice with the galactosylated peptide CII256–270, but not with the corresponding non-glycosylated peptide, showed protection against development of CIA. The protective effect was much more pronounced when the rat peptide sequence (Glu266) was used instead of the mouse peptide (Asp266).[28] The difference could be an effect of a higher affinity of the rat sequence in binding to A^q, or an indication of position 266 being a T-cell contact point.[17] Moreover, a recent report showed that administration of the galactosylated peptide CII256–270 in complex with A^q significantly reduced both disease progression and severity in mice with ongoing chronic relapsing arthritis.[29]

The specificity of the T-cell hybridomas obtained in CIA has been addressed with regard to the structural features of the carbohydrate moiety,[26,27] as well as the side chain[30–32] of galactosylated hydroxylysine 264 in the immunodominant epitope CII256–270. However, little is known about the importance of the peptide (e.g., side chains and amide bonds) for the recognition by the carbohydrate dependent hybridomas. The role of peptide side chains in events of biological recognition is traditionally studied by performing an alanine-scan, that is, by replacing the amino acids, one by one, with alanine. More recently peptide libraries, which provide more information if appropriately designed, have been used.[33–35] The importance of the backbone amides for recognition can be studied using amide bond isosteres, which have altered hydrogen bonding and electrostatic properties. Successful incorporations of amide bond mimetics could also circumvent many of the poor characteristics of peptides used as drugs, that is, poor bioavailability, rapid enzymatic degradation and lack of selectivity.[36] The trans-ethylenic isostere $\Psi[(E)CH{=}CH]$ lacks both the hydrogen bond donor and acceptor possibilities of a normal amide bond, but shows close approximation in geometrical terms with the trans amide bond.[37] This isostere has been incorporated in the immunodominant peptide from hen egg white lysozyme (HEL) and the analogue was found to bind to the A^k MHC class II molecule and also to stimulate HEL-specific T-cell clones. In contrast, the more flexible and charged aminomethylene isostere $\Psi[CH_2NH_2^+]$ did not even bind to A^k when incorporated in the same peptide.[38] In a similar study, when the aminomethylene isostere was introduced into peptides known to bind to the MHC class II molecules DR1 and DR4, binding was lost for most isosteric positions, possibly indicating the need for hydrogen bonding to backbone amides, or reflecting the increased flexibility.[39] The requirement of hydrogen bonding of the backbone amides for binding to the MHC explains difficulties to identify peptidomimetics useful for therapy. However, E^d restricted peptides that are reduced and N-methylated in appropriate positions have been found to induce tolerance, indicating that they were bound to E^d and possessed T-cell reactivity.[40]

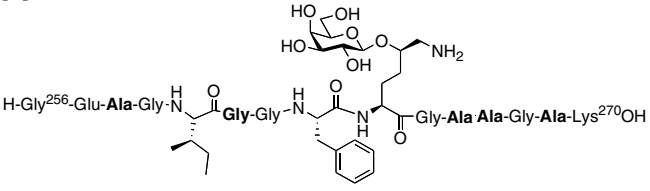
In this paper, we investigate the requirements of the side chains and backbone in the immunodominant core of

the CII derived glycopeptide associated with development of CIA. Alanine substituted glycopeptides were used to study the role of side chains in binding to A^q class II MHC molecules and in recognition by the T-cells, while a methylene ether isostere which replaced one of the amide bonds was used to highlight the role of the peptide backbone. The methylene ether isostere ($\Psi[CH_2O]$) was first introduced in 1986[41] and offers a polar, flexible and proteolytically resistant alternative to the amide bond. Moreover, the C^{i}_{α}–C^{ii}_{α} distance of the $\Psi[CH_2O]$ pseudopeptide unit is calculated to be 3.7 Å,[41] which is remarkably close to the distance (3.8 Å) of unmodified peptides. None of the normal hydrogen bonding acceptor/donor properties of an amide bond are maintained, making it an excellent isostere for studying hydrogen bonding interactions. The results reported in this, and related future studies, could lead to successful attempts to interfere with the T-cell response in RA and thereby to development of drugs based on glycopeptide mimetics.

2. Results and discussion

To investigate the role of the side chains of the glycosylated immunodominant CII peptide for T-cell recognition, the responses towards two alanine substituted glycopeptides were studied. The first being a heavily alanine and glycine substituted glycopeptide, henceforth referred to as **Gal-polyAla** (Table 1). Within residues 258–269 four amino acids (residues 258, 266, 267 and 269) have been replaced by alanine, while the naturally occurring alanine 261 was exchanged for a glycine. For obvious reasons the MHC A^q P1 and P4 anchor residues isoleucine 260 and phenylalanine 263 were kept intact, just as the galactosylated hydroxylysine 264. Despite our earlier findings, identifying the minimal glycopeptide epitope required to generate a strong T-cell response to be CII260–267,[18] the peptide was kept as the longer immunodominant counterpart CII256–270. This was done in order to prevent possible loss in MHC binding due to the simultaneous replacement of many amino acids (we suspected that not only the P1 and P4 anchors contribute to the MHC binding). The second alanine substituted glycopeptide has the normal sequence of the CII epitope at positions 259–273 except for introduction of alanine at position 266. This glycopeptide is henceforth referred to as **Gal266Ala**. Position 266 not only distinguishes rat from mouse CII (glutamic acid in rat, aspartic acid in mice) but several observations indicate it as interacting with both MHC and the T-cell receptor. For instance, when a polyclonal T-cell line was generated from mice immunised with rat CII we showed that the T-cell line recognised both the galactosylated immunodominant CII peptide of the heterologous (rat) and the mouse sequence.[28] However, an approximately 100 times higher concentration was needed of the mouse glycopeptide **m259-273Gal** to give a response of the same magnitude as for the rat peptide. Even though **m259-273Gal** is known to bind weaker to A^q class II MHC molecules this suggests position 266 to be a T-cell contact. Further support was provided by analysing the T-cell response on the clonal level, which revealed that 50% of the tested T-cell hybridomas responded to the mouse glycopeptide **m259-273Gal** while 50% did not. When either the mouse or the heterologous non-glycosylated CII256–270 peptide was bound covalently into the peptide binding pocket of the A^q molecules, all

Table 1. Modified glycopeptides and reference peptides used to study the side-chain requirements for stimulation of T-cell hybridomas

Alanine substituted glycopeptides		
Gal-polyAla		rCII 256–270, Gal-Hyl 264, P258A, A261G, E266A, Q267A, P259A
Gal266Ala	H-Gly^{259}-Ile-Ala-Gly-Phe-Hyl(Gal)-Gly-**Ala^{266}**-Gln-Gly-Pro-Lys-Gly-Glu-Thr^{273}-OH	rCII 259–273, Gal-Hyl 264, E266A
Modified CLIP peptides		
GalCLIP	Ac-Ser^{88}-Gln-Met-Arg-Met-Ala-Hyl(Gal)-Pro-Leu-Leu-Met-Arg-Pro^{100}-NH_2	Ii88–100, Gal-Hyl 94
CLIP	Ac-Ser^{88}-Gln-Met-Arg-Met-Ala-Thr-Pro-Leu-Leu-Met-Arg-Pro^{100}-NH_2	Ii88–100
Reference peptides		
m259-273Gal	H-Gly^{259}-Ile-Ala-Gly-Phe-Hyl(Gal)-Gly-**Asp**-Gln-Gly-Pro-Lys-Gly-Glu-Thr^{273}-OH	mCII 259–273, Gal-Hyl 264
r259-273Gal	H-Gly^{259}-Ile-Ala-Gly-Phe-Hyl(Gal)-Gly-**Glu**-Gln-Gly-Pro-Lys-Gly-Glu-Thr^{273}-OH	rCII 259–273, Gal-Hyl 264
r259-273	H-Gly^{259}-Ile-Ala-Gly-Phe-Lys-Gly-Glu-Gln-Gly-Pro-Lys-Gly-Glu-Thr^{273}-OH	rCII 259–273

rCII, rat collagen type II; mCII, mouse collagen type II; Ii, invariant chain; CLIP, class II-associated invariant chain peptide.

T-cell hybridomas reactive with the heterologous naked peptide recognised both peptides.[42] This would imply inefficient presentation as the reason for the weaker T-cell response towards the mouse peptide.

The **Gal-polyAla** and **Gal266Ala** glycopeptides, as well as the other glycopeptides used in this study, were prepared using solid-phase peptide synthesis. The peptides were purified by reversed-phase HPLC and characterised with mass spectrometry. Despite the many substitutions in **Gal-polyAla**, the glycopeptide retained almost all binding to the A^q MHCII molecule; in a binding assay using purified A^q a twofold higher concentration of **Gal-polyAla** was needed to reach the same binding as for the native rat peptide **r259-273**. This correlates well with another study in which polyalanine substituted peptides, with anchor residues intact, retained binding to DR1 class II MHC molecules. In this study it was concluded that the majority of the peptide side chains were not required for high affinity binding to DR1 molecules.[43]

The two substituted peptides and the reference peptides (cf. Table 1) were tested for T-cell recognition by incubation with splenic A^q expressing antigen presenting cells and one of five different collagen-specific T-cell hybridomas. Earlier studies using deoxygenated galactose analogues to address the fine specificity for the galactose moiety[26] made it possible to subdivide the galactose-specific hybridomas into five different groups. One hybridoma from each group was finally chosen as representative members for this investigation. Recognition of the MHC–peptide complex by the T cell hybridomas resulted in IL2 secretion into the media, which was quantified using an antibody/streptavidin–Eu^{3+} based assay, an 'Eu^{3+}LISA.' As expected all T-cell hybridomas responded to the positive control, the glycosylated rat peptide **r259-273Gal**, while none recognised the non-glycosylated peptide with lysine at position 264 (**r259-273**), thereby confirming the sugar dependence of the recognition (Fig. 2A). Three out of the five hybridomas, HNC1, HCQ.10 and HM1R.2 from carbohydrate specificity groups 1, 2 and 4, respectively, responded to the penta-substituted **Gal-polyAla** peptide just as well as to the positive control **r259-273Gal**. This means that these three hybridomas do not need any side-chain residue for stimulation, besides the galactosylated hydroxylysine 264. In accordance with these data the same hybridomas also responded just as well, or even better, to the single alanine substituted peptide **Gal266Ala** and to the mouse glycopeptide **m259-273Gal**, having aspartic acid in position 266 (Fig. 2B). On the other hand, hybridomas HCQ.3 and 22a1-7E from groups 3 and 5, respectively, did not respond to **Gal-polyAla** (Fig. 2A). These two hybridomas are obviously dependent on binding to one or more of the removed side chains. Looking at the response towards **Gal266Ala** and **m259-273Gal** refines the picture. The two hybridomas recognise neither of these two glycopeptides, revealing position 266 to be a T-cell contact point.[44] However, it should be pointed out that this does not prove position 266 to be the only side-chain contact point for these two T-cell hybridomas. In summary, studies using the substituted glycopeptides **Gal-polyAla** and **Gal266Ala** showed that there are T-cells that do not need any side chain besides galactosylated hydroxylysine 264 to respond, while others also require the glutamic acid in position 266 as a T-cell contact point. These results correlate very well with a study of peptides presented by A^b MHC class II molecules,[45] which show 89% sequence identity with A^q in the peptide binding site. A^b restricted T-cells often recognise the side chain of the residues found in the P7 pocket (preferably Asn), corresponding to position 266 when peptides from CII are presented by A^q. Furthermore, our findings that three out of five hybridomas were independent of all side chains except galactosylated hydroxylysine 264, and could thus not distinguish between rat and mouse collagen, explain the cross-reactivity of the T-cell line obtained after immunization of mice with rat CII.

The results obtained with the **Gal-polyAla** glycopeptide imply that there could be T-cell hybridomas that respond to highly modified glycopeptides as long as the anchor residues for the P1 and P4 pockets of A^q, and the galactosylated hydroxylysine moiety, are retained in the correct positions. To further test this hypothesis we designed a completely different glycopeptide based on the peptide backbone of the self peptide CLIP, which was modified by introducing a galactosylated hydroxylysine at a central position (henceforth referred to as **GalCLIP**, Table 1). The CLIP peptide, consisting of amino acids 88–100 from the invariant chain, was chosen because it occupies and stabilises MHC class II molecules before they are loaded with antigen in the peptide binding groove. Since no crystal structure was available for the binding of the CLIP peptide to A^q, the design of **GalCLIP** was instead based on the structure of CLIP bound to the A^b molecule. This approach was justified since A^q and A^b show 89% sequence identity in the peptide binding site. In the CLIP A^b complex methionine 90 occupies the P1 pocket of A^b and alanine 93 is located in the P4 pocket. If CLIP is located in the same manner in A^q then threonine 94 should be replaced by a galactosylated hydroxylysine in order to allow proper interactions with the T-cell receptor. When binding to A^q was tested in an inhibition assay using whole cells, CLIP was bound approximately equivalent to that of the reference **r259-273**, while **GalCLIP** bound with approximately threefold lower affinity. The **GalCLIP** peptide and the non-glycosylated CLIP reference were tested for recognition by the same T-cell hybridomas as for the two alanine substituted glycopeptides (Fig. 2A). Unfortunately, none of the five hybridomas recognised **GalCLIP**. The lack of response from hybridomas HCQ.3 and 22a1-7E is not surprising since these hybridomas are dependent on glutamic acid 266 in the CII derived glycopeptides. The finding that neither of the three side-chain independent hybridomas HNC.1, HCQ10 and HM1R.2 responded could be due to several reasons. For instance, the galactosylated hydroxylysine could have been placed in the wrong position, or the large side chains in **GalCLIP** peptide could cause steric hindrance in binding of the T-cell receptor. In order to investigate these possibilities further, the coordinates for **CLIP** bound to A^b were used to place **CLIP** in a structural model of the A^q molecule (created by comparative (homology) modelling

with A^b as template, to be published) and energy minimised. Rat collagen peptide **Gal260-267** (Table 2) was also docked into the model of A^q and energy minimised. The P4 anchor positions of the **CLIP** peptide (Ala 93) and **Gal260-267** (Phe 263), as well as the galactosylated hydroxylysine 264 and threonine 94 moieties in these two A^q-bound glycopeptides, overlaid well, suggesting that the galactose moiety is correctly located in **GalCLIP** (Fig. 3A). When looking at **CLIP** bound to the A^q (Fig. 3B), it can be seen that the large side chain of arginine 91 protrudes out of the A^q-binding site. Since alanine 261 is located in this position in the **Gal260-267** A^q complex steric hindrance from arginine 91 most likely explains why **GalCLIP** is not recognised by the T-cell hybridomas. The results suggest that it is essential not to have too large groups pointing up towards the T-cell receptor other than the galactosylated hydroxylysine moiety, and glutamine 266, at least for some hybridomas.

Apart from binding to side chains, T-cell receptors could potentially also bind to the backbone amides of A^q-restricted glycopeptides, even though these are extensively hydrogen bonded to the class II MHC molecule. As a first step in studies of backbone–TCR interactions a methylene ether isostere[46] was incorporated instead of the amide bond between isoleucine 260 and alanine 261 in the minimal collagen glycopeptide **Gal260-267** (Table 2). The methylene ether isostere was chosen since it has been found not to have a major effect on the secondary structure of the substituted peptide,[46] and since it lacks the hydrogen bonding possibilities of amides. Two isosteric glycopeptides were synthesised, one with a free N-terminus and the other with an acetylated N-terminus (**iso** and **Ac-iso**, respectively, Table 2). Both were prepared as C-terminal amides to avoid potential interference between a charge at the C-terminus of the peptides and A^q and/or the T-cell receptor. When the two glycopeptides were tested for binding to A^q in a competition assay with biotinylated CLIP peptide **iso** was found to lack binding. In contrast, **Ac-iso** bound but required approximately 20 times higher concentration to reach the same binding as the native rat peptide **r259-273**. The lack of binding of **iso** to A^q is most likely due to the positive charge at the N-terminus of **iso**. For **Ac-iso** the reduced binding may reflect that the Ile260-Ala261 amide bond is involved in hydrogen bonding to A^q. The two isosteres were then tested for their ability to stimulate a panel of seven T-cell hybridomas (Fig. 4). The hybridomas were chosen from four groups with different selectivity for the galactose moiety. In addition,

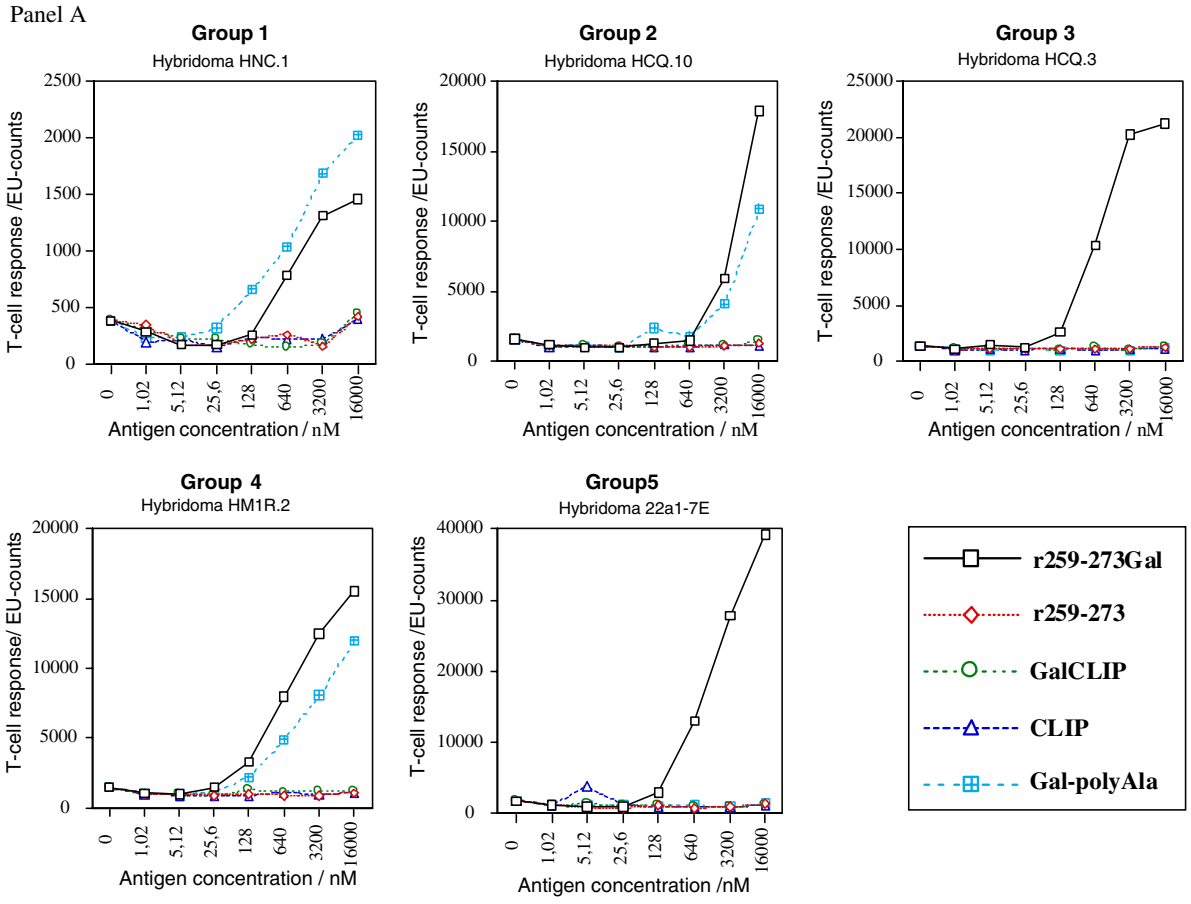


Figure 2. Responses of five different T-cell hybridomas towards side-chain altered glycopeptides. Representatives from each of five groups of hybridomas, previously mapped with different fine specificity for the galactose moiety of hydroxylysine 264, were chosen for this analysis. Secretion of interleukin 2 (IL2) due to recognition of the A^q–glycopeptide complex by T-cell hybridomas was quantified in an IL2-antibody assay and detected by streptavidin–Eu^{3+} (Eu^{3+}LISA). The values are represented as Eu^{3+} counts. (A) The response to the heavily substituted glycopeptide **Gal-polyAla** and **GalCLIP**. (B) Response to **Gal266Ala** which contains a single substitution at position 266.

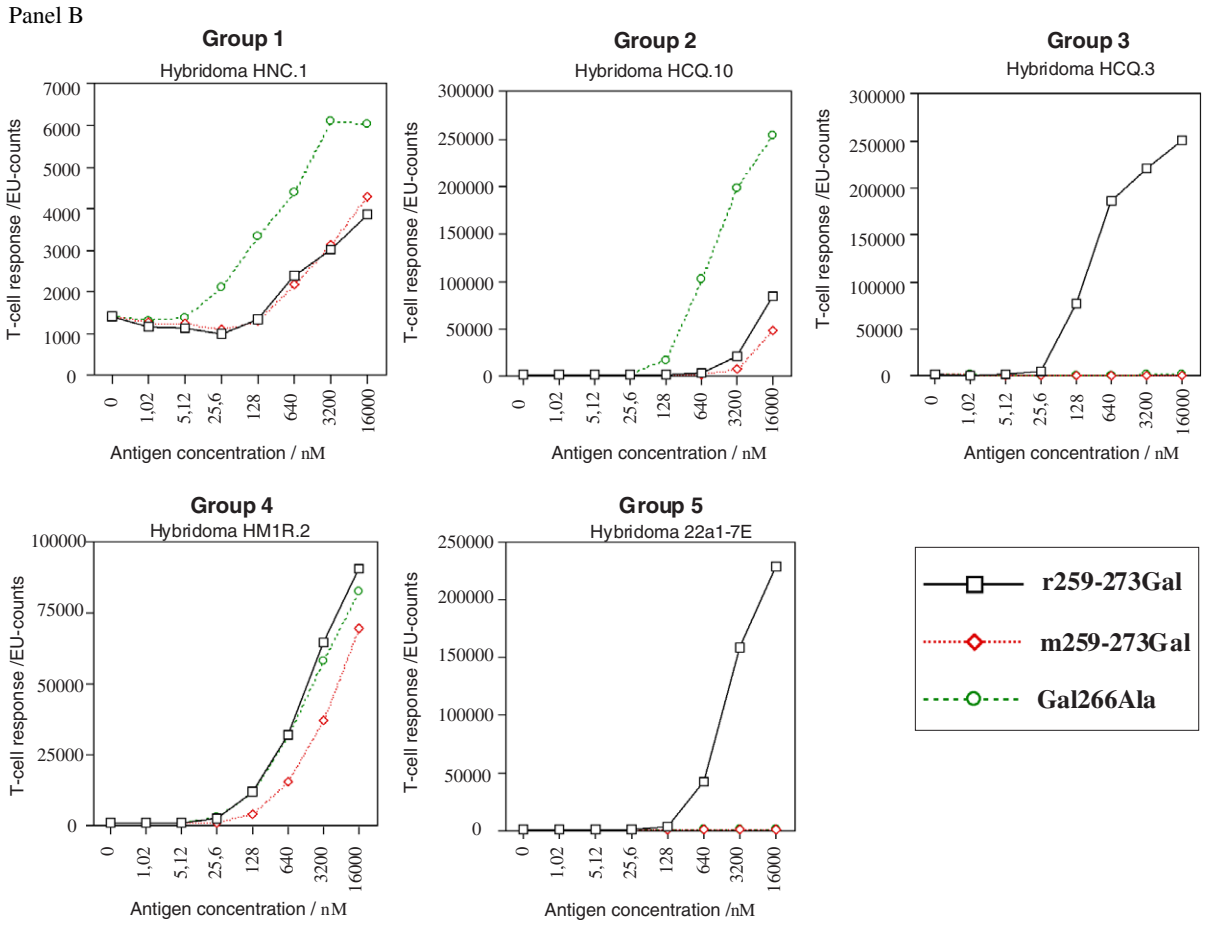
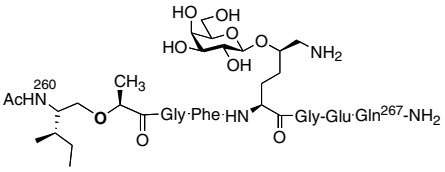
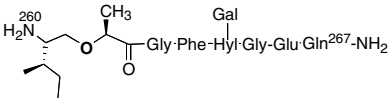
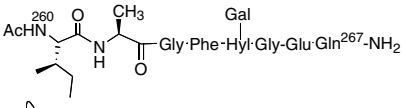


Figure 2. (*continued*)

Table 2. Isostere glycopeptides and reference peptides used to study the amide bond requirements for stimulation of T-cell hybridomas

Isostere peptides		
Ac-iso		rCII 260–267, Gal-Hyl 264, Ile-ψ[CH_2O]-Ala, C-NH_2, N-Ac
iso		rCII 260–267, Gal-Hyl 264, Ile-ψ[CH_2O]-Ala, C-NH_2
Reference peptides		
Gal260-267		rCII 260–267, Gal-Hyl 264, C-NH_2, N-Ac
dCII		Denaturated rCII, glycosylated and non-glycosylated 264
r259-273Gal	H-Gly^{259}-Ile-Ala-Gly-Phe-Hyl(Gal)-Gly-Glu-Gln-Gly-Pro-Lys-Gly-Glu-Thr^{273}-OH	rCII 259–273, Gal-Hyl 264
r259-273	H-Gly^{259}-Ile-Ala-Gly-Phe-Lys-Gly-Glu-Gln-Gly-Pro-Lys-Gly-Glu-Thr^{273}-OH	rCII 259–273

rCII, rat collagen type II.

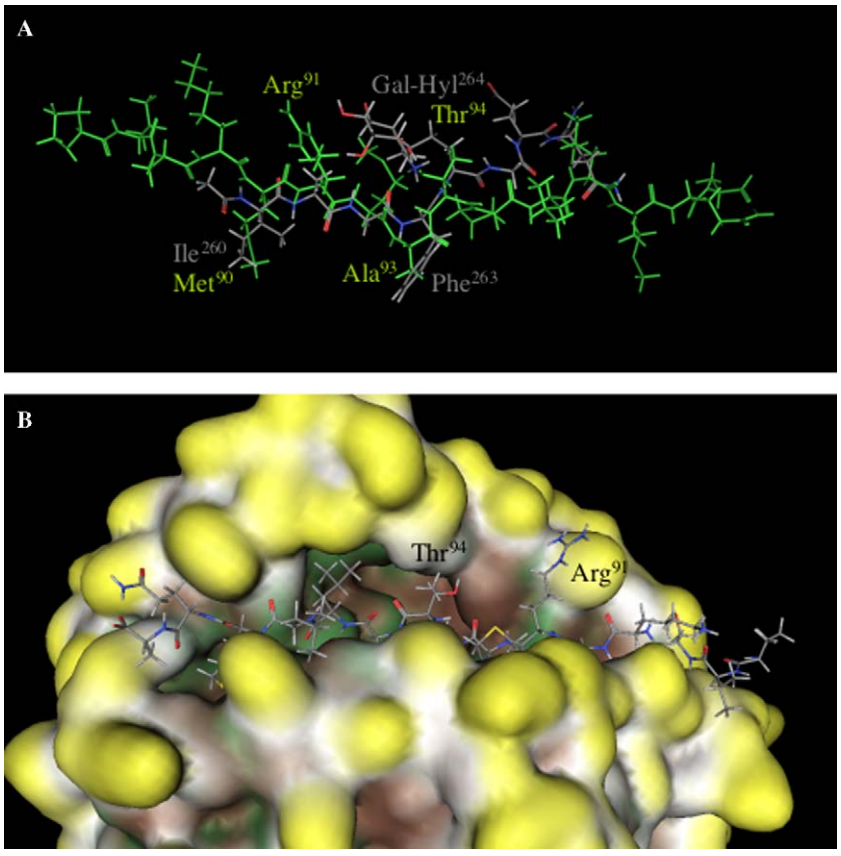


Figure 3. (A) Comparison of the conformations of the **CLIP** peptide and the **Gal260-267** glycopeptide, in the binding site of the modelled A^q molecule. The anchor positions, as well as threonine 94 and the galactosylated hydroxylysine 264, which overlap well, are indicated. (B) The **CLIP** peptide has been located in the modelled A^q-molecule and the complex has then been energy minimised. Both arginine 91 and threonine 94, the latter of which was selected for replacement by a galactosylated hydroxylysine residue, point up towards a potential T-cell receptor. The molecular A^q surface is coloured yellow, dark green and dark brown for exposed, hydrophilic and hydrophobic residues, respectively. In order to provide a better view of the residues extending from the binding site of A^q the peptide is oriented with its N-terminus to the right in (B). In line with common practice the N-terminus is located to the left in (A).

a hybridoma known to be specific for lysine at position 264 (HCQ.4) was included as control. As expected none of the hybridomas recognised the non-acetylated isostere **iso** since it lacks binding to the A^q MHC class II molecule. The acetylated isostere **Ac-iso**, on the other hand, was recognised by both group two and three hybridomas, but 100–1000 times higher concentrations than for **Gal260-267** were required to reach a maximum T-cell response. This finding reflects the weaker binding of **Ac-iso** to A^q and could also indicate a weak interaction between the T-cell receptor and the Ile260-Ala261 amide bond in **Gal260-267**. The hybridomas from groups one and four did not respond to **Ac-iso** at the tested concentrations. A comparison of how the different T-cell hybridomas recognise the isostere **Ac-iso** and the alanine substituted peptides **Gal-polyAla** and **Gal266Ala** provided more detailed structural information on how the hybridomas recognise the A^q restricted CII glycopeptide. Those hybridomas (HNC1 and HM1R2) not responding to **Ac-iso**, and thereby indicating a need for hydrogen bonding to the Ile^{260}-Ala^{261} amide bond, are also the hybridomas that responded towards the two alanine substituted peptides, that is, those that are independent of all side chains except the galactosylated hydroxylysine. On the other hand HCQ.3, which requires glutamic acid 266, responds to **Ac-iso**. There is also one hybridoma, HCQ.10, that responds to the two alanine substituted peptides, as well as to the isostere, and thus seems to be independent of both side chains and the backbone in the peptide.

Based on the data presented in this paper, and in a previous study detailing the fine-specificity for the galactose moiety,[26] we hypothesise that the TCRs of the different hybridomas recognise the galactose on hydroxylysine from different sides (Fig. 5). The TCRs of the two hybridomas from group three bind to the galactose, hydroxylysine and down to glutamic acid 266 in the C-terminal part of the peptide. The TCRs of the three hybridomas belonging to groups one and four are 'independent' of the peptide side chains but instead bind to the amide bond between Ile260 and Ala261 in the N-terminal part of the peptide. It is possible that the galactose moiety then displays different epitopes to the hybridomas in order to allow formation of critical interactions. This should be facilitated by the flexibility of the glycosidic bond to hydroxylysine. In contrast, hybridomas HCQ10 from group two are independent of peptide side chains, as well as of the Ile-Ala amide bond. These differences in recognition pattern, including the ability

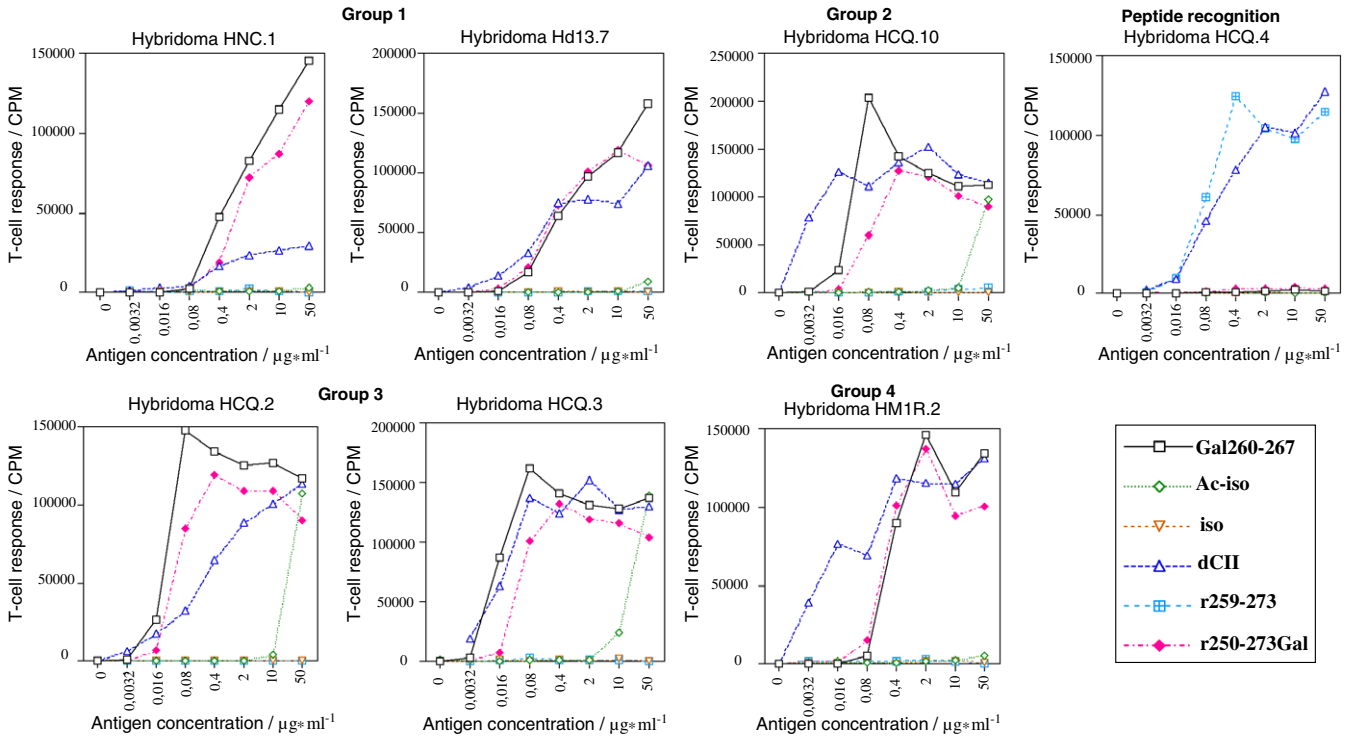


Figure 4. Response of seven different T-cell hybridomas towards glycopeptides containing an amide bond isostere. The hybridomas were chosen from different groups previously mapped with different fine specificity for the galactose moiety of hydroxylysine 264. Secretion of IL-2 into the media due to recognition of an A^q–peptide complex by a hybridomas was measured in a 3H-thymidine based proliferation assay using an IL-2 sensitive CTLL clone.

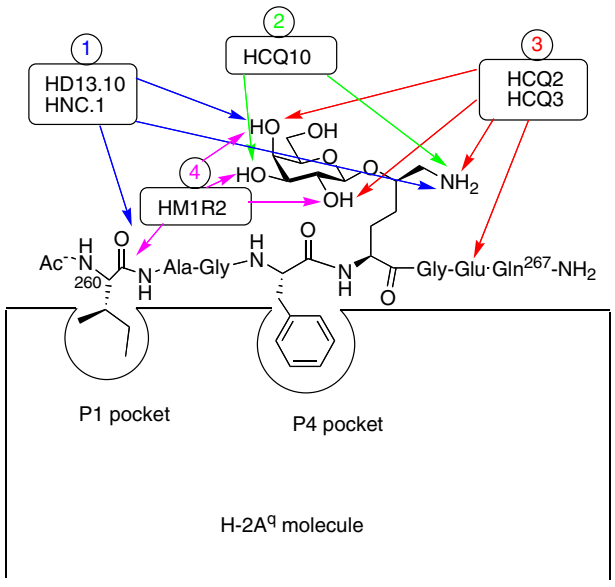


Figure 5. Overview of the T-cell receptor contact points for four groups of hybridomas which recognise the glycopeptide **Gal260-267** presented by A^q MHC class II molecules with different fine specificity. Apart from galactosylated hydroxylysine 264, glutamic acid 266 was the only side chain in the peptide moiety that was found to be of importance for any of the hybridomas. Three hybridomas from different groups require the amide bond between isoleucine 260 and alanine 261, in order to respond. We hypothesise that the TCR binds from one or the other side of the galactose moiety when recognising glutamic acid 266 or the amide bond.

to distinguish between self (mouse) and non-self (rat) derived glycopeptides, could possibly allow development of different strategic approaches for controlling T-cell auto-reactivity. The finding of none or merely one side-chain dependence (position 266), besides galactosylated hydroxylysine 264, for T-cell recognition opens up for future and more drastic modifications of the peptide part in an altered peptide ligand approach. It is possible that introduction of peptidomimetics could be used in order to alter the agonist/antagonist activity and/or the pharmacokinetic properties. We are presently extending the inclusion of amide bond isosteres in the CII peptide backbone to complete the study of peptide dependence of the hybridomas and to further refine the T-cell interaction model.

3. Experimental

3.1. General methods and materials

Analytical reversed-phase HPLC was performed on a Kromasil C-8 column (250 × 4.6 mm, 5 μm, 100 Å), eluted with a linear gradient of MeCN (0–100%, alternatively 0–80%, over 60 min) in H_2O; both eluents containing 0.1% TFA. A flow rate of 1.5 mL/min was used and detection was at 214 nm. Preparative reversed-phase HPLC was performed on a Kromasil C-8 column (250 × 20 mm, 5 μm, 100 Å), with the same eluents, a flow rate of 11 mL/min and detection at 214 nm. The

T-cell hybridomas used in this study originate from DBA/1 (HDB.2, HM1R.2) and C3H.Q (HCQ.2, HCQ.4, HCQ.10) mice and were established earlier.[17,22,47,23] Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 5–10% FCS, Hepes, penicillin, glutamine, streptomycin and β-mercaptoethanol. CTLL cells were cultured prior use with an additional 0.3% X63-IL2 for expansion and starved of IL2 two days before use in determination of T-cell hybridoma response.

3.2. Gal-Hyl and isostere building blocks

Hydroxylysine (Hyl) was protected in several steps to give (5*R*)-N^{α}-(flouoren-9-ylmethoxycarbonyl)-N^{ε}-benzyloxycarbonyl-5-hydroxy-L-lysine allyl ester.[48] This derivative was used in a silver silicate promoted glycosylation with acetobromogalactose to yield a protected Gal-Hyl building block.[49] After removal of the allyl ester (5*R*)-N^{α}-(fluoren-9-ylmethoxycarbonyl)-N^{ε}-benzyloxycarbonyl-5-*O*-(2,3,4,6-*tetra*-*O*-acetyl-β-D-galactopyranosyl)-5-hydroxy-L-lysine (Gal-Hyl) was obtained ready for use in solid-phase peptide synthesis. Ile Ψ[CH_2O]Ala building block, (2*S*)-2-((2*S*,3*S*)-2-azido-3-methyl-pentoxi)proponic acid, was synthesised in two steps from aminoalcohol and (*R*)-2-chloropropionic acid,[46] with the azidogroup instead of Fmoc protected amines like in standard amino acid building blocks.

3.3. General procedure for solid-phase peptide synthesis

The peptides and glycopeptides were synthesised in a manually operated reactor or on a Pioneer™ Peptide Synthesis System (Applied Biosystems, Netherlands), using standard solid-phase peptide synthesis methodology. The longer (glyco)peptides (Tables 1 and 2) were synthesised on a Tentagel-S-PHB resin (Rapp Polymere, Germany) with the first amino acid (the C-terminal one) pre-attached to the support. This provided these (glyco)peptides as C-terminal carboxylic acids after cleavage from the resin. The shorter (glyco)peptides on the other hand were synthesised on a Tentagel-S-NH_2 resin (Rapp Polymere, Germany) where the linker Fmoc-2,4-dimethoxy-4′-(carboxymethyloxy)-benzhydrylamine (Rink) was first coupled to the resin. This rendered (glyco)peptides as C-terminal amides after cleavage from the resin. N^{α}-Fmoc amino acids carrying standard side-chain protective groups (Bachem, Switzerland and Neosystem S.A., France, 4 equiv), as well as the Rink-linker (4 equiv), were coupled to the resin in dimethylformamide (DMF), that was predistilled and used immediately or stored for a short time over 3 Å molecular sieves. In the manually operated reactor, diisopropyl carbodiimide (DIC, 3.9 equiv) in the presence of 1-hydroxy-benzotriazole (HOBt, 6 equiv) was used as coupling reagent. The completion of the reaction was monitored by the naked eye using bromophenol blue as indicator.[50] Alternatively, coupling reactions taking place in the Pioneer™ Peptide Synthesis System utilised HBTU 0.5 M and DIPEA 0.5 M as coupling reagents with UV monitoring, all according to the manufacturer's instructions. (5*R*)-N^{α}-(fluoren-9-ylmethoxycarbonyl)-N^{ε}-benzyloxycarbonyl-5-*O*-(2,3,4,6-*tetra*-*O*-acetyl-β-D-galactopyranosyl)-5-hydroxy-L-lysine[49] (Gal-Hyl, 1.2–1.4 equiv) and the methylene ether isostere building block (2*S*)-2-((2*S*,3*S*)-2-azido-3-methyl-pentoxi)proponic acid[46] (Ile Ψ[CH_2O]Ala, 1.7 equiv) were coupled in DMF using DIC (1.2–1.4 equiv) and 1-hydroxy-7-aza-benzotriazole (1.8–2.1 equiv) as coupling reagents for 24 h. Removal of Fmoc protective groups after each coupling cycle was performed using 20% piperidine in DMF. The azido group of the Ile Ψ[CH_2O]Ala building block was reduced to free amine by treatment with tin (II) chloride in the presence of thiophenol and triethylamine.[46,51] The reduction was monitored by IR spectroscopy directly on the solid phase. The reduced, resin bound product was washed with 20% piperidine in DMF to remove any remaining complexed tin-salts that could otherwise prevent the following acetylation. After completing the synthetic sequence, the N-terminus of the shorter peptides was acetylated for 1 h using Ac_2O/DMF (1:2). Cleavage and deprotection of the peptides was performed with TFA/H_2O/thioanisole/ethanedithiol (35:2:2:1) for 3 h at 40 °C. Following repeated concentration from HOAc the (glyco)peptides were precipitated from Et_2O and the crude products freeze-dried. Deacetylation of glycopeptides was performed using NaOMe (20 mM) in MeOH for approximately 2 h, after which the solution was neutralised by addition of 10% HOAc in MeOH and concentrated. Purification on reversed-phase HPLC and freeze-drying gave the homogeneous compounds (Tables 1 and 2). The identity of the (glyco)peptides was confirmed by MS and in addition by NMR spectroscopy for the isosteric glycopeptide **Ac-iso** and **Gal260-267** (Tables 3 and 4).

3.4. Peptide binding assays

The binding of peptides to A^q MHC class II molecules was performed using either of two competition assays, flow cytometry analysis (**GalCLIP**, **CLIP**) or a time-resolved fluoroimmunoassay (**iso**, **Ac-iso**, **Gal260-267**), as described before.[18] In short, increasing concentrations of the competitor peptides **GalCLIP** and **CLIP** were incubated for 2.5 h at 37 °C with a fixed concentration of biotinylated CLIP peptide (5 μM) and M12Q 14-7 cells transfected with A^q. After washing to remove excess peptide, the cells were stained with 0.2 μL streptavidin–phycoerythrin (SAPE), which bound to the biotinylated CLIP peptide. The phycoerythrin (PE) dye was detected by flow cytometry analysis using the FACSort (Becton–Dickinson, San Jose, CA) and Becton–Dickinson software. The IC_{50} for each peptide was calculated from the gated mean fluorescence by comparing with the positive control (no inhibiting peptide) after subtracting the negative control (no biotinylated CLIP peptide). In order to study the binding of **iso**, **Ac-iso**, **Gal260-267**, increasing concentrations of the glycopeptides were incubated for 60 h at room temperature with a fixed concentration (2.0 μM) of biotinylated CLIP peptide and affinity purified A^q molecules (0.1 μM). A^q–peptide complexes were captured in a 96-well microtitre plate pre-coated with the monoclonal antibody Y3-P. Complexes between A^q and biotinylated CLIP peptide were quantified using the dissociation-enhanced lanthanide fluoroimmunoassay (DELFIA®) kit

Table 3. ^{1}H NMR chemical shifts for glycopeptide **Ac-iso** recorded in aqueous buffer containing 10% D_2O,[a] and a 500 MHz spectrometer

Residue	NH	Hα	Hβ	Hγ	Others
NH_2-C-terminal					7.54, 7.08 ($CONH_2$)
Gln^{267}	8.44	4.27	2.09, 1.95	2.34[b]	7.47, 6.81 ($CONH_2$)
Glu^{266}	8.25	4.32	2.08, 1.92	2.41[b]	
Gly^{265}	7.98	3.89[b]			
Gal					4.42 (H1), 3.51 (H2), 3.62, 3.90 (H3,H4), 3.67 (H5), 3.75 (H6)
Hyl^{264}	8.44	4.27	2.01, 1.75	1.59[b]	4.01 (Hδ), 3.17, 2.97 (Hε), 7.59[b] (2NHε)
Phe^{263}	8.12	4.53	3.10, 3.01		7.23 (2H), 7.35, 7.31 (2H)
Gly^{262}	8.31	3.89[b]			
'Ala^{261}'		3.95	1.28		
'Ile^{260}'	8.03	3.82	1.55	0.84 (CH_3), 1.39, 1.07	0.81 (Hδ) 3.51, 3.61 (CH_2O)
Ac-N-terminal					1.96

[a] Measured at 298 K, H_2O calibrated to 4.76.
[b] Degeneracy has been assumed.

Table 4. ^{1}H NMR chemical shifts for glycopeptide **Gal260-267** recorded in aqueous buffer containing 10% D_2O,[a] and a 500 MHz spectrometer

Residue	NH	Hα	Hβ	Hγ	Others
NH_2-C-terminal					7.46, 6.79 ($CONH_2$)
Gln^{267}	8.45	4.22	2.07, 1.91	2.30[b]	7.52, 7.07 ($CONH_2$)
Glu^{266}	8.39	4.22	2.00, 1.89	2.22	
Gly^{265}	7.73	3.84			
Gal					4.36 (H1), 3.47 (H2), 3.58, 3.86 (H3,H4) 3.63 (H5), 3.71 (H6)
Hyl^{264}	8.36	4.23	1.66, 1.96	1.53	3.96 (Hδ), 2.92, 3.12 (Hε)
Phe^{263}	8.06	4.55	3.03		7.29 (1H), 7.24 (2H), 7.20 (2H)
Gly^{262}	8.27	3.84			
Ala^{261}	8.39	4.24	1.31		
Ile^{260}	8.09	4.06	1.76	0.85 (CH_3), 1.39, 1.12	0.79 (H*δ*)
Ac-N-terminal					1.96

[a] Measured at 298 K, H_2O calibrated to 4.76.
[b] Degeneracy has been assumed.

system based on the time-resolved fluoroimmunoassay technique with europium-labelled streptavidin (Wallac, Turku) according to the manufacturer's instructions. The IC_{50} for each glycopeptide was calculated from the mean fluorescence.

3.5. Determination of T-cell hybridoma response

The response of each T-cell hybridoma, i.e., IL-2 secreted on incubation of the hybridoma with antigen presenting spleen cells and increasing concentrations of glycopeptides (isosteric, side-chain altered and reference (glyco)peptides), was determined in a standard assay using the T-cell clone CTLL,[52] or an IL2 antibody assay. In brief, 5×10^4 T-cell hybridomas were co-cultured with 5×10^5 syngeneic, spleen cells and antigen in a volume of 200 μL in 96-well flat-bottom microtitre plates. After 24 h, 100 μL aliquots of the supernatants were removed and frozen to kill any transferred T-cell hybridomas. For the study of the T-cell response to the isostere peptides **iso** and **Ac-iso**, IL-2 sensitive CTLL T-cells (1×10^5/ml, 100 μl/well) were added to the thawed supernatant. The CTLL cultures were incubated for 24 h, after which they were pulsed with 1 μCi ^{3}H-thymidine and incubated for an additional 15–18 h. The cells were harvested on glass fibre sheets in a Filtermate TM cell harvester (Packard Instruments, Meriden, CT) and the amount of radioactivity was determined in a matrix 96™ Direct Beta Counter (Packard). All experiments were performed in duplicate. For measurement of IL-2 secretion in the study of side-chain altered peptides, Maxisorp Nunc-Immunoplates were coated with the capture antibody JES6-1A12 (BD Pharmingen, Cat. No. 554424) for 2 h. The plates were blocked with 2% BSA after which 50 μL of the culture supernatants was incubated for 2 h. A biotinylated JES6-5H4 antibody (BD Pharmingen, Cat. No. 554426) was used as detection antibody. A system based on streptavidin–EU^{3+} (DELFIA, Wallac, Finland) was used, referred to as Eu^{3+}LISA. The bound EU^{3+} was released through an enhancer solution (DELFIA, Wallac, Finland) and detected by a heterogeneous time-resolved flourometric assay using a Victor/1420 Multilabel counter (Perkin-Elmer), according to the manufacture's instructions. The values are represented as EU^{3+} counts.

3.6. Docking studies of Gal260-267 in comparison with a CLIP peptide in a model of A^q

The crystal coordinates of the CLIP peptide in the MHC II molecule A^b (PDB Accession code 1MUJ)[53] were placed into a new model of the A^q molecule (I. Andersson et al., personal communication) and energy minimised using MOE[54] force field Amber 94. The glycosylated minimal peptide Gal260-267 was built in SYBYL (version 7.0)[55] and the 3D structure used for docking was generated in Corina.[56] Docking of Gal260-267 in Gold (version 2.2)[57] was performed with

the radius of the binding site set to 17 Å. Goldscore was used as the fitness function with hydrogen bonding and van der Waals parameters set to 2.5 and 4.0, respectively. For each genetic algorithm run, a default maximum number of 100,000 operators were performed on a population of five islands of 100 individuals and terminated after 30 runs. The operator weights for crossover, mutation and migration were set to 95, 100 and 0, respectively. Out of the 30 resulting conformations the best-ranked conformation selected visually based on the known features of peptide–A^q interactions, that is, an extended backbone, peptide anchor positions pointing down in A^q binding cleft and galactose moiety pointing upwards, was used in a second docking run to generate the final conformation.

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